

LISTING OF CLAIMS

Claim 1 (Currently Amended): A method for detecting pathogenic mycobacteria in clinical specimens, said method comprising steps of:

- (a) clarifying the clinical specimens from containment ~~including mucus~~ by conventional methods,
- (b) treating the processed clinical specimens obtained in step (a) with ~~the a~~ modified lysis buffer to inactivate ~~the~~ live pathogenic mycobacteria to make the process safe for the user,
- (c) extracting genomic DNA from the processed clinical specimen obtained from step (b) using a modified method to increase the yield and quality of DNA,
- (d) designing sequence of SEQ ID No. 4 from the DNA obtained in step (c) for specific detection of pathogenic mycobacteria, said designed sequence comprising of selected intergenic region of SEQ ID. No. 3, a flanking region containing a portion of the gene mmaA1 of SEQ ID No. 1 and a portion of gene mmaA2 of SEQ ID No. 2 of the DNA obtained in step (c),
- (e) designing and synthesizing a set of specific oligonucleotide primers of SEQ ID No. 5, which is the forward primer and SEQ ID No. 6, which is the reverse primer for Polymerase Chain Reaction (PCR) amplification of SEQ ID No. 4,
- (f) developing a PCR amplification process for specific amplification of SEQ ID No. 4 of (d), said process comprising using the specific oligonucleotide primers ~~of~~ designed and synthesized in step (e) for detecting presence of pathogenic mycobacteria in the clinical specimens, and
- (g) analysing the amplified PCR product by restriction fragment length polymorphism (RFLP) analysis for differentiation of the species of the pathogenic mycobacterium for a quick assessment of HIV co-infection.

Claim 2 (Currently Amended): A method as claimed in claim 1, wherein the designed SEQ ID No. 4 is ~~having~~ has a sequence as follows:

5'GAGGTGTAATGCCTTTCCGGACCCTAGGTGGCCTTTCGGTGCTTGCA
G
GAACGCACCGATGCTTCCCCCTCCCCGCATGCTCGAGGCATGCTATCCG
A
TACAGGGCCGCCGCACTAAACCGCGATCGAATTTGCCCAGGTCAGGGA
A
CGGATATGAGCGGACGAG3'

5'TGGATCCGTTGACCATGAGGTGTAATGCCTTTCCGGACCCTAGGTGGC
CTTTCGGTGCTTGCAACGCAACCGATGCTTCCCCCTCCCCGCATGC
TCGAGGCATGCTATCCGATACAGGGCCGCCGCACTAAACCGCGATCGA
ATTTGCCCAGGTCAGGGAACGGATATGAGCGGACGAGCTACTTGGTCA
TGGTGAACCTGGGCGACGTTGATTAGGCCTCTGCGGAAGCGCTCCGCGCA
TCCGGTCAGATAGTGCATGAAGTTGTTGTAGACCTCTTCGGACTGTACG
GCGATGGCGCGTTCGCGGGCAGCCTGTAGGTTGGCGGCCCATGCATCG
AGAGTCCGTGCGTAGTGGAATTC 3'.

Claim 3 (Currently Amended): A method as claimed in claim 1, wherein[[,]] the clinical specimen is selected from the group consisting of sputum, gastric lavage, cerebrospinal fluid, blood, tissue biopsies, ~~or~~ bone marrow aspirates and other body fluids or tissues.

Claim 4 (Currently Amended): A method as claimed in claim 1, wherein[[,]] clarification of ~~specimen~~ the specimens in the step (a) from the contaminants (~~live organisms other than mycobacteria and mucus~~) is carried out by adding to said specimens a digestion decontamination mix containing mild alkali, NaOH, tri sodium citrate and a

mucolytic agent and guanidinium isothiocyanate in the range of about 0.4-2.5 M followed by concentrating the ~~specimen~~ specimens from centrifugation.

Claim 5 (Currently Amended): A method as claimed in claim 4, wherein[[,]] the digestion decontamination mix containing mild alkali, NaOH, tri sodium citrate and a mucolytic agent and guanidinium isothiocyanate in the range of about 0.5-2.0 M.

Claim 6 (Currently Amended): A method as claimed in claim 1, wherein[[,]] the DNA in step (c) is extracted from the treated clinical specimen using a modified lysis buffer by inclusion of ingredients comprising guanidinium isothiocyanate in a range of about 0.5-8 M, Tris.Cl pH 7.6 is in a ~~the~~ range of about 20-100 mM, N lauryl Sarcosyl is in a range of about 0.5-2% by weight of the buffer, EDTA is in ~~the~~ a range of about 0.1-20 mM, β -Mercaptoethanol is in ~~the~~ a range of about 1-25 mM and NH_4COOH NaCl is ~~in the~~ range present in an amount of about ~~0.3M-1M~~ 0.2M; and purifying the DNA to improve yield by thorough precipitation by organic solvents.

Claim 7 (Currently Amended): A method as claimed in claim 6, wherein guanidinium isothiocyanate is about 4M, Tris-HCl pH 7.6 is about 50 mM, N lauryl Sarcosyl is about 1% by weight of the buffer, EDTA is about 1 mM, β -Mercaptoethanol is about 10 mM and NH_4COOH NaCl is about ~~0.7~~ 0.2M.

Claim 8 (Currently Amended): A method as claimed in claim 6, wherein [[,]] the organic solvents are selected from the group ~~comprising~~ consisting of a phenol/chloroform mixture and chloroform.

Claim 9 (Currently Amended): A method as claimed in ~~claims 1 and 6~~ claim 1, wherein[[,]] the genomic DNA yield is increased in the range of about 25 to 50%.

Claim 10 (Currently Amended): A method as claimed in claim 9, wherein[[,]] the genomic DNA yield is increased in the range of about 30 to 40%.

Claim 11 (Currently Amended): A method as claimed in claim 1, wherein[[,]] high yielding amplification of DNA in step (f) is achieved by the modified Touch ~~down~~ Down PCR cycling conditions, said conditions comprising steps of providing an initial high annealing temperature in the range of about 62-72°C followed by lowering of temperature in the range of about 0.1-1°C per PCR cycle for the first 10-25 cycles, ~~which is the touch down step to an optimum annealing temperature of about 56-62°C for another 30 PCR cycles~~ then subsequently carrying out 30 PCR cycles at an optimum annealing temperature of about 56-62°C.

Claim 12 (Currently Amended): A method as claimed in claim ~~13~~ 1, wherein[[,]] high yielding amplification of DNA is achieved by modified Touch Down PCR cycling conditions, said conditions comprising steps of providing an initial high annealing temperature of about 70°C followed by lowering of temperature of about 0.8°C per PCR cycle for about first 14 cycles to about 58°C for another 25 PCR cycles.

Claim 13 (Currently Amended): A method as claimed in claim 1, wherein[[,]] the oligonucleotide primers capable of amplification of intergenic region of SEQ ID No. 4 for detection of pathogenic Mycobacteria in clinical specimens are selected from the group consisting of:

- a. 5' TGGATCCGTTGACCATGAGGTGTAATG 3' (SEQ ID No. 5), which is the forward primer[[,]], and
- b. ~~5' GGAATTCCACTACCGGACTCTC 3'~~
5' GGAATTCCACTACGCACGGACTCTC 3' (SEQ ID No. 6), which is the reverse primer.

Claim 14 (Currently Amended): A method as claimed in claim 1, wherein[,] the length of oligomeric primers is between 5 and 100 bases.

Claim 15 (Currently Amended): A method as claimed in ~~claims~~ claim 1, wherein[,] the modified lysis buffer provides a cleaner preparation of the DNA.

Claim 16 (Currently Amended): A method as claimed in claim 1, wherein[,] treatment with the modified lysis buffer containing 4M guanidinium isothiocyanate inactivates the live mycobacteria to make the procedure safer fro the operator.

Claim 17 (Currently Amended): A diagnostic kit for the detection of pathogenic mycobacteria in clinical specimens, comprising primers selected from the group consisting of:

- a. 5' TGGATCCGTTGACCATGAGGTGTAATG 3' (SEQ ID No. 5), which is the forward primer, and
- b. 5' GGAATTCCACTACGCACGGACTCTC 3' (SEQ ID No. 6), which is the reverse primer.

Claim 18 (New): A method as claimed in claim 1, wherein the contaminant clarified in step (a) comprises mucus and/or live organisms other than mycobacteria.